N-Terminal Protein Characterization by Mass Spectrometry Using Combined Microscale Liquid and Solid-phase Derivatization

Heinz Nika,[1,2] Ruth Hogue Angeletti,[1,2] and David H. Hawke[3], *

[1]*Laboratory for Macromolecular Analysis and Proteomics and* [2]*Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461, USA; and* [3]*MD Anderson Cancer Center, The University of Texas, Houston, Texas 77030, USA*

A sample-preparation method for N-terminal peptide isolation from protein proteolytic digests has been developed. Protein thiols and primary amines were protected by carboxyamidomethylation and acetylation, respectively, followed by trypsinization. The digest was bound to ZipTip$_{C18}$ pipette tips for reaction of the newly generated N-termini with sulfosuccinimidyl-6-[3′-(2-pyridyldithio)-propionamido] hexanoate. The digest was subsequently exposed to hydroxylamine for reversal of hydroxyl group acylation, followed by reductive release of the pyridine-2-thione moiety from the derivatives. The thiol group-functionalized internal and C-terminal peptides were reversibly captured by covalent chromatography on activated thiol sepharose leaving the N-terminal fragment free in solution. The use of the reversed-phase supports as a reaction bed enabled optimization of the serial modification steps for throughput and completeness of derivatization. The use of the sample-preparation method was demonstrated with low picomole amounts of in-solution- and in-gel-digested protein. The N-terminal peptide was selectively retrieved from the affinity support. The sample-preparation method provides for throughput, robustness, and simplicity of operation using standard equipment available in most biological laboratories and is anticipated to be readily expanded to proteome-wide applications.

KEY WORDS: peptide isolation, protein digest, reversed-phase support, covalent chromatography, gel-separated protein

INTRODUCTION

Protein terminal regions are widely recognized as essential determinants of diverse cellular functions, such as protein degradation, localization, and membrane interaction.[1,2] Technologies aimed at the characterization of positional domains, a subfield of functional proteomics called N-terminomics, involve the isolation of terminal peptides from digests prepared from individual proteins or from fractionated complex proteomics mixtures for subsequent structural characterization by tandem mass spectrometry (MS/MS). Such efforts are greatly facilitated by the ensuing complexity reduction and the high sequence specificity at the protein termini, resulting in an increased throughput of analysis and enhanced success rate of protein identification.[3]

Method developments targeting N-terminal peptides by negative or positive selection have been, thus far, of main interest (reviewed by Nakazawa et al.[4]). Negative selection strategies rely on primary amines protection, preceded by thiol alkylation, followed by proteolytic digestion of the derivatives and depletion of the internal and C-terminal peptides via the newly generated (neo)-N-termini.[5–8] One method advocated for this purpose involved protein acetylation, tryptic digestion of the derivatives, biotinylation of the internal, and C-terminal peptides, followed by their removal on streptavidin sepharose.[5] In a variation of this approach, the internal and C-terminal peptides were scavenged by *N*-hydroxysuccinimide-activated sepharose, leaving the N-terminal peptide free in solution.[6] As a result of the lengthy individual reaction steps, nearly 2 days were required to complete this procedure. Positive N-terminal peptide selection relies on protein ε-amine guanidination, followed by biotinylation of the N-terminal using sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate. After protein tryptic digestion, the N-terminal peptide was retained on avidin sepharose, from which the fragment was released by oxidative cleavage of the biotinamido-linked disulfide bond.[7] In a variation of this approach, tryptic digests of the selectively biotinylated proteins were submitted to performic oxidation, followed by capture of the internal and C-terminal peptides by p-phenylenediisothio-

*Address correspondence to: David H. Hawke, Dept. of Pathology, MD Anderson Cancer Center, 7435 Fannin St., Houston, TX 77054, USA (Phone: 713-834-6096; Fax: 713-834-6113; E-mail: dhawke@ mdanderson.org).

cyanate (DITC) glass, leaving the oxidized N-terminal peptides in the flow-through fraction.[8] Although the sulfonation of N-terminal fragments facilitated MS/MS data interpretation, issues persist as to their diminished detectability in matrix-assisted laser desorption/ionization (MALDI)-time-of-flight-MS.[9]

In the strategy reported here, the thiol and primary amino group protection was allowed to proceed in a one-pot reaction scheme or on gel-separated protein, followed by trypsinization. The internal and C-terminal peptides were scavenged via their N-termini on N-hydroxysuccinimide (NHS) activated sepharose, leaving the unbound N-terminal fragment in the flow-through fraction.[6] Alternatively, the N-terminal peptide can be selected on dendrimer aldehyde-functionalized polymers and retrieved from the matrix by centrifugal filtration.[10] However, these depletion procedures consume ~18 h, limiting the overall throughput of the methods. To remedy this situation, we introduced a masked thiol group into the internal and C-terminal peptides using sulfosuccinimidyl-6-[3′-(2-pyridyldithio)-propionamido] hexanoate (Sulfo-LC-SPDP) as an acylation reagent, followed by reduction of the derivatives' terminal 2-pyridinyldithiol groups. The internal and C-terminal peptides thiolated in this manner were reversibly captured on activated thiol sepharose; the N-terminal fragment remained free in solution. Less than 2 h were required to complete the affinity purification step. The bound material may be reductively retrieved from the affinity support for further manipulation, e.g., phosphorylation-site determination, which is not possible when relying on depletion methods thus far described in the literature.

MATERIALS AND METHODS

Trifluoroacetic acid (TFA), Sulfo-NHS ester of acetic acid (Sulfo-NHS acetate), Sulfo-LC-SPDP, hydroxylamine hydrochloride, Bond-Breaker Tris (2-carboxyethyl) phosphine (TCEP) solution (0.5 M), Handee spin columns (0.8 mL internal volume), and GelCode Blue Stain Reagent were obtained from Pierce (Rockford, IL, USA). N-Octyl glucoside (OGS) was obtained from Roche Diagnostics (Indianapolis, IN, USA). Sodium phosphate dibasic dodecahydrate was purchased from Fluka (Ronkonkoma, NJ, USA). Methanol and acetonitrile were from Burdick & Jackson (Muskegon, MI, USA). Iodoacetamide, sodium phosphate monobasic monohydrate, 1 M Tris-HCl/0.1 M EDTA buffer (pH 8.0), sodium carbonate, ammonium bicarbonate, rat serum albumin (RSA), human serum albumin (HSA), bovine carbonic anhydrase, horse heart myoglobin, bovine β-lactoglobulin, human angiotensin I acetate salt hydrate [DRVYIHPFHL, mass-to-charge ratio (m/z) 1296.5], and human [Glu[1]] fibrinopeptide B (EGVNDNEEFFAR, m/z 1570.5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Activated thiol sepharose 4B and urea (ultra-pure grade) were from GE Healthcare (Piscataway, NJ, USA). The α-cyano-4-hyroxycinnamic acid preparation was from Agilent Technologies (Palo Alto, CA, USA). ZipTip$_{C18}$ pipette tips (0.6 μL bed vol) and ZipTip$_{μ-C18}$ pipette tips (0.2 μL bed vol) were purchased from Millipore (Billerica, MA, USA). Trypsin (modified sequencing grade) was purchased from Promega (Madison, WI, USA). Polyacrylamide gels (Criterion Precast gel, 1 mm, 10%) were from Bio-Rad Laboratories (Hercules, CA, USA).

Protein In-Gel Carboxyamidomethylation/Acetylation/ Proteolytic Digestion

GelCode Blue stained bands from 10 to 50 pmoles RSA, loaded onto the gels, were destained twice with 200 μL of 25 mM ammonium bicarbonate in 50% aqueous acetonitrile for 30 min at 37°C. Bands were then briefly dried in a SpeedVac and then incubated for 15 min at 37°C in 100 μL of 2 mM TCEP/25 mM ammonium bicarbonate; the supernatant was then removed. The gel bands were incubated in 100 μL of 20 mM iodoacetamide in 25 mM ammonium bicarbonate for 30 min at 37°C; the supernatant was then discarded. Bands were washed three times with 200 μL water for 15 min and briefly dried in a SpeedVac. Bands were dehydrated for 10 min in 100 μL acetonitrile and then briefly dried in a SpeedVac. Sulfo-NHS acetate (40 μL of 20 mM in 50 mM sodium phosphate buffer, pH 8.0) was then added. After incubation for 2 h at 37°C, the gel bands were washed three times with 200 μL water for 15 min at 37° and subsequently three times with 200 μL of 25 mM ammonium bicarbonate for 15 min at 37°C. Bands were briefly dried in a SpeedVac and then dehydrated for 10 min in 100 μL acetonitrile and briefly dried. Bands were reswollen at room temperature in 20 μL of 25 mM ammonium bicarbonate/0.01% OGS, supplemented with Promega-modified trypsin to an enzyme:substrate ratio of 1:10. After 20 min, 40 μL of 25 mM ammonium bicarbonate was added, and the digestion continued for 18 h at 37°C with agitation. After incubation, the supernatant was removed. TFA (50 μL of 0.1%) was added. The gel bands were incubated for 45 min at 37°C. The combined extracts were reduced in volume to 35 μL and acidified by addition of 5 μL of 10% TFA before sample immobilization on ZipTip$_{C18}$ pipette tips, performed according to the procedure described below. Peptides were desalted by passing 100 μL of 0.1% TFA over the resin and typically eluted in 50% acetonitrile/ 0.1% TFA/0.01% OGS before MALDI-MS or submitted to sequential solid-phase derivatization as described below.

One-Pot Protein Carboxyamidomethylation/Acetylation-Tryptic Digestion

Model protein (10–200 pmoles) was reduced in 30 μL of a 10 mM sodium phosphate/4 M urea solution (pH 8.0), supplemented with 2.5 mM TCEP and 0.01% OGS. After 20 min incubation at 55°C, 10 uL of a 150 mM iodoacetamide solution prepared in 10 mM sodium phosphate/4 M urea/0.01% OGS was added to a final concentration of 30 mM, and the incubation continued for 30 min at 37°C in the dark. A Sulfo-NHS acetate solution (10 μL of 60 mM in 10 mM sodium phosphate/4 M urea/0.01% OGS urea) was added to a final concentration of 10 mM. The mixture (50 μL) was incubated for 20 min at 55°C. The derivatized protein sample was conditioned for tryptic digestion by buffer exchange (BEX) using Zeba Spin Desalting Columns with a 7 kDa molecular weight cut-off (MWCO). Ammonium bicarbonate/1 M urea/0.005% OGS solution, pH 8.0 (400 μL of 50 mM) was applied to the resin bed, followed by centrifugation for 1 min at 1500 g. This step was repeated five times. The protein sample (50 μL) was applied to the spin columns and allowed to absorb completely on the resin. Water (20 μL) was then applied to the resin bed, followed by centrifugation for 2 min at 1500 g. An aqueous trypsin solution (5 μL) was added to the recovered sample to give a substrate:enzyme ratio of 20:1. The digestion, allowed to proceed overnight at 37°C, was arrested by addition of 5 μL of 25% TFA. The sample volume was reduced to 40 μL by SpeedVac evaporation. The digest was subsequently bound to ZipTip reversed-phase supports, desalted by passing 100 μL of 0.1% TFA in 10 μL aliquots over the resin, and processed for MALDI-MS or solid-phase peptide derivatization as described below.

Sample Adsorption on C18 Reversed-Phase Support

ZipTip$_{C18}$ pipette tips and ZipTip$_{\mu\text{-}C18}$ pipette tips were wetted six times with 10 μL methanol, followed by six washes with 10 μL of 0.1% TFA . Model peptides (10–100 pmoles) were prepared in 0.5% TFA/0.01% OGS, placed in 10 μL aliquots into 0.5 mL microfuge tubes, and subjected to 10 sample aspiration/dispense cycles. The tips were washed three times with 10 μL of 0.1% TFA to removed unbound material. For high-volume sample enrichment (typically 40–100 μL), the peptide solutions were aspirated sequentially in 10 μL aliquots and dispensed into a 0.5 ml microfuge tube. The partially stripped peptide solution was then transferred back in this step-wise mode to the original sample tube. This alternating load/dispense sample enrichment was repeated five times to maximize peptide binding. The ZipTip pipette tips were then washed 10 times with 10 μL of 0.1% TFA. To avoid resin dewetting, ZipTips loaded with test peptides or digests were

processed immediately for derivatization or kept in temporary storage in 10 μL of 0.1% TFA, aspirated onto the supports from 60 μL solvent. The tips, left immersed in solvent, may be stored at −20°C for several days.

General Procedure for Solid-Phase Peptide Derivatization

Reagent (10 μL) was aspirated twice onto the ZipTip$_{C18}$ pipette tips or ZipTip$_{\mu\text{-}C18}$ pipette tips and dispensed to waste. Reagent (10 μL) was then loaded onto the tips from 60 μL that had been placed into 0.5 ml microfuge tubes. The tips were left immersed in the reagents during incubation and subsequently desalted, as specified below. The tips were eluted in 5–10 μL of 50% acetonitrile/0.1% TFA/0.01% OGS, of which 1 μL was typically used for MALDI-MS analysis. ZipTip$_{\mu\text{-}C18}$ pipette tips were eluted in matrix supplemented with 0.1% TFA/0.01% OGS onto the MALDI target.

On-Column Acetylation

Sulfo-NHS acetate was dissolved in 50 mM sodium phosphate buffer, pH 8.0, to a final concentration of 20 mM. ZipTip$_{C18}$ pipette tips were loaded with 10 μL reagent and incubated for 20 min at 55°C. The ZipTips were then washed 10 times with 10 μL aliquots of 0.1% TFA. Sample processing for MALDI-MS was as described above.

On-Column Acylation with Sulfo-LC-SPDP

A 20 mM sodium phosphate buffer solution, pH 8.0, was supplemented with 20 mM Sulfo-LC-SPDP. ZipTip$_{C18}$ pipette tips were incubated in 10 uL reagent for 30 min at room temperature, washed 10 times with 10μL 0.1% TFA, and processed for MALDI-MS, as described above.

On-Column O-Deacylation

Hydroxylamine hydrochloride was dissolved in 1 M sodium carbonate, pH 9.4, to a final concentration of 2%. ZipTip$_{C18}$ pipette tips were incubated in 10 μL reagent for 15 min at 37°C and then washed 10 times with 10 μL of 0.1% TFA and then five times with 10 μL water. Sample processing for MALDI-MS was as described above.

On-Column Disulfide-Bond Reduction

A 20 mM sodium phosphate solution, pH 8.0, was supplemented with 5 mM TCEP. Reagent (10 μL) was loaded onto the ZipTip$_{C18}$ pipette tips. After incubation for 15 min at 37°C, the tips were washed 10 times with 10 μL of a 2 mM aqueous EDTA solution and then 10 times with 10 μL of 0.1% TFA. Samples were processed for MALDI-MS, as described above.
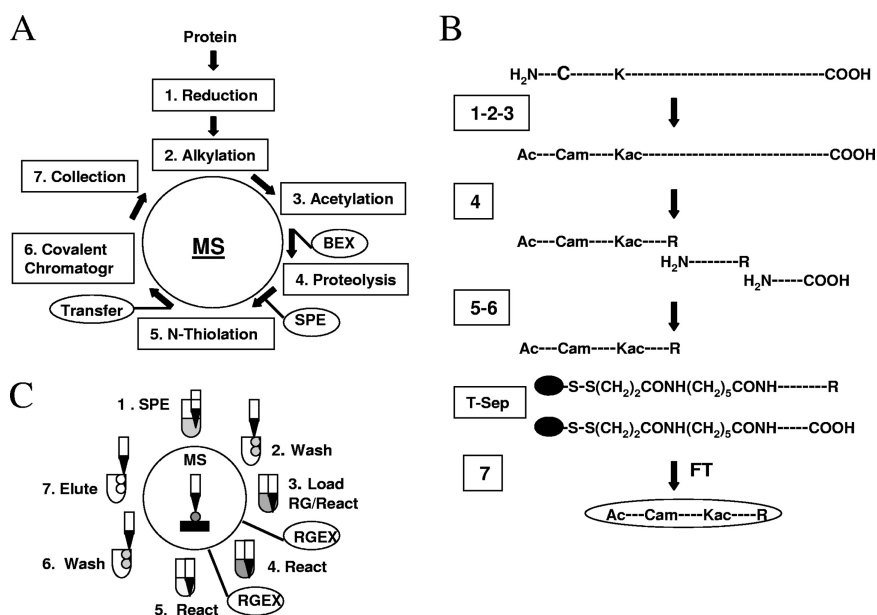
FIG. 1

(A) Flow chart of sample preparation for N-terminal peptide isolation. The reaction steps are numbered and highlighted with boxes. Whole protein is reduced and carboxyamidomethylated (Steps 1 and 2), followed by acetylation (Step 2) in a one-pot reaction sequence. After BEX, the protein is digested with trypsin, followed by adsorption of the digest on ZipTip$_{C18}$ pipette tips (SPE). Subsequent processing on the solid phase, denoted as N-thiolation (Step 5), includes reaction with Sulfo-LS-SPDP, *O*-deacylation to reverse unwanted hydroxyl group transesterification, and reduction of the conjugates' terminal 2-pyridinyl disulfide group. After sample transfer to activated thiol sepharose, the thiol group-functionalized internal and C-terminal peptides are scavenged by covalent chromatography (Chromatogr; Step 6). The N-terminal peptide remains free in solution and is collected in the flow-through fraction (Step 7). Optionally, the bound peptides can be reductively released from the support and used for further structural investigation, e.g., phosphorylation-site determination.[11] (B) Schematic representation of the reaction path. Ac and Cam denote acetyl and carboxyamidomethyl group, respectively. T-Sep and FT denote activated thiol sepharose and flow-through, respectively. (C) Schematic representation of solid-phase sample handling steps. After solid-phase extraction (SPE), the analyte is desalted, followed by the reagent (RG)-loading step. Note that reagents are then sequentially exchanged on the reaction bed (RGEX), eliminating intermittent sample purification, and that the ZipTip$_{C18}$ pipette tip is left immersed in reagent during incubation. The reaction cycle is concluded by a solvent wash before product elution, MALDI-MS analysis, and transfer to activated thiol sepharose.

Consecutive On-Column Derivatization: Sample Preparation at the Peptide Level

The NHS-ester reaction, *O*-deacylation, and disulfide-bond reduction were combined into the sequential reaction scheme, as depicted schematically in Fig. 1C. The intermittent desalting steps before reagent loading were as specified for the individual chemistries described above. Each reagent loading step was preceded by flushing the desalted tips twice to waste with 10 μL reagent. During incubation, the tips were left immersed in reagent. After reaction with Sulfo-LC-SPDP and subsequent *O*-deacylation, the desalted ZipTips can be stored for several days at −20°C in 10 μL 0.1% TFA, leaving the ZipTips immersed in solvent. After the reduction step, care must be taken to remove the reductant thoroughly from the support by sequentially passing 100 μL aqueous 2 mM EDTA and 100 μL of 0.1% TFA in 10 μL aliquots over the resin. The final reaction

products were eluted in10 μL of 50% acetonitrile/0.1% TFA/0.01% OGS into 40 μL of 50 mM sodium phosphate/2 mM EDTA/2 M urea/0.01% OGS (pH 8.0), used as coupling buffer for covalent chromatography (see following section).

Covalent Chromatography

Activated thiol sepharose (1 g) was allowed to swell for 10 min in 10 ml water and then washed by vacuum filtration with a total of 150 ml water added in 15 ml aliquots, suspended in 10 ml of 10% aqueous ethanol, and stored refrigerated until use. The Handee spin column used as reaction vessel was washed with 200 μL acetonitrile by centrifugation at 1500 rpm for 1 min, a centrifugation speed and time setting that was used throughout the protocol. A gel slurry (100 μL of 75%) was placed into the spin column and centrifuged. The affinity medium was resus-

pended in 200 μL of 50 mM sodium phosphate/2 mM EDTA, pH 8.0, or in 50 mM Tris-HCl/5 mM EDTA, pH 8.0, followed by centrifugation. The spin column was sealed with the plastic plugs supplied by the manufacturer. The digests (and test peptides) conditioned for covalent chromatography were eluted from the ZipTip$_{C18}$ pipette tip with 10 μL of 50% acetonitrile/0.1% TFA/0.01% OGS into 40 μL thiol-coupling buffer composed of 50 mM sodium phosphate/2 mM EDTA/2 M urea/0.01% OGS (pH 8.0) or of 50 mM Tris-HCl/5 mM EDTA/2 M urea/0.01% OGS (pH 8.0). The mixture was transferred to the medium pellet, which was then gently shaken to facilitate resuspension. The spin column was capped, inserted into a 1.5 mL microfuge tube, and secured with Parafilm. After end-over-end incubation for 1 h at room temperature in a rotary mixer, the unbound material (i.e., the N-terminal fragment) was displaced from the resin by centrifugation. The affinity medium was washed consecutively with 50 μL thiol-coupling buffer, 50 μL of 60% aqueous acetonitrile/0.1% TFA, and 50 μL of 80% aqueous acetonitrile/0.1% TFA. The combined flow-through fractions were reduced in volume by SpeedVac evaporation to 35 μL and acidified with 5 μL TFA to a final concentration of 1%. The isolates were recovered on ZipTip$_{C18}$ pipette tips and desalted with 100 μL of 0.1% TFA, passed over the resin in 10 μL aliquots. Peptides were eluted from the support with 10 μL of 50% acetonitrile/0.1% TFA/0.01% OGS before MALDI-MS analysis. Low-level amounts of peptide were enriched on micro ZipTips, washed 10 times with 10 μL aliquots of 0.1% TFA, and deposited in matrix containing 0.1% TFA onto the MALDI target. To retrieve the disulfide-bound material, the affinity medium was washed with 50 μL thiol-coupling buffer; the flow-through was discarded. The coupling buffer (50 μL), supplemented with 5 mM TCEP, was added to the resin bed and allowed to react with end-over-end mixing for 30 min at room temperature. In some experiments, 10 μL of 300 mM iodoacetamide (prepared in coupling buffer) was added and the incubation continued for 30 min. Peptides were displaced from the affinity support by centrifugation. The resin was washed with 50 μL reductant and subsequently, with the organic solvents, as described above. The combined fractions were reduced in volume to 35 μL and acidified with 5 μL 10% TFA to a final concentration of 1%. The material was bound to ZipTip$_{C18}$ pipette tips, washed 10 times with 10 μL of 0.1% TFA, and stored immersed in 0.1% TFA until further use.

MS

A Voyager-DE STR (Applied Biosystems, Foster City, CA, USA) was used and operated in the reflector mode at an accelerating voltage of 20 kV, 67% grid voltage, and 250 ns

extraction delay time. Laser intensity was typically set at 1400–1600. Spectra were acquired using 80 laser shots/spectrum, and spectra sampled from eight different positions were averaged. The analyte was prepared on the target in the dried droplet mode using α-cyano-4-hydroxycinnamic acid as matrix. For peptide fragmentation, a 4800 Proteomics Analyzer (Applied Biosystems) was used and operated in the MS and MS/MS mode; typical laser power for MS was ~3700; for MS/MS, ~4500. Usually 1000–2000 shots were acquired for MS, 2000; 20,000 for MS/MS.

RESULTS AND DISCUSSION
Reaction Scheme

As illustrated in Fig. 1A and B, the sample-preparation scheme is initiated by protein disulfide bond reduction, followed by carboxyamidomethylation of the liberated thiols (Steps 1 and 2). The protein is then subjected to acetylation to block primary amino groups (Step 3). The one-pot reaction mixture is BEX; the recovered fraction submitted to proteolysis (Step 4). The digest is extracted on ZipTip$_{C18}$ pipette tips (SPE) and reacted in situ with Sulfo-LC-SPDP targeting the neo N-termini, subsequently with hydroxylamine for reversal of unwanted hydroxyl group acylation, followed by reductive release of the pyridine-2-thione moiety from the derivatives. The serial solid-phase reaction cycle, collectively denoted as N-thiolation (Step 5), is halted by a clean-up step, followed by product elution and sample transfer to thiol-activated thiol sepharose for depletion of the thiol-functionalized internal and C-terminal peptides by covalent chromatography (Step 6). The N-terminal peptide remains free in solution and is collected in the flow-through fraction (Step 7). Optionally, the disulfide-bonded material can be retrieved from the affinity support for further manipulation, e.g., phosphorylation-site determination.

Sample Handling

The solid-phase derivatization format used in our study offers some notable advantages over the classical in solution-based methods, the predominant sample-preparation technique in current proteomic studies.[11] In brief, the solid-phase strategy exploits the sample-preconcentration effect, allowing the reaction to proceed in situ, in general, at higher efficiency and faster kinetics than in solution.[11,12] Protein digests can be enriched effectively on the solid phase from dilute solutions, in which chemical reactions inherently proceed at slow reaction rates. As demonstrated in this report and in earlier communications, the solid-phase reaction format proved particularly advantageous for combining distinct chemistries into serial reaction schemes.[13,14]

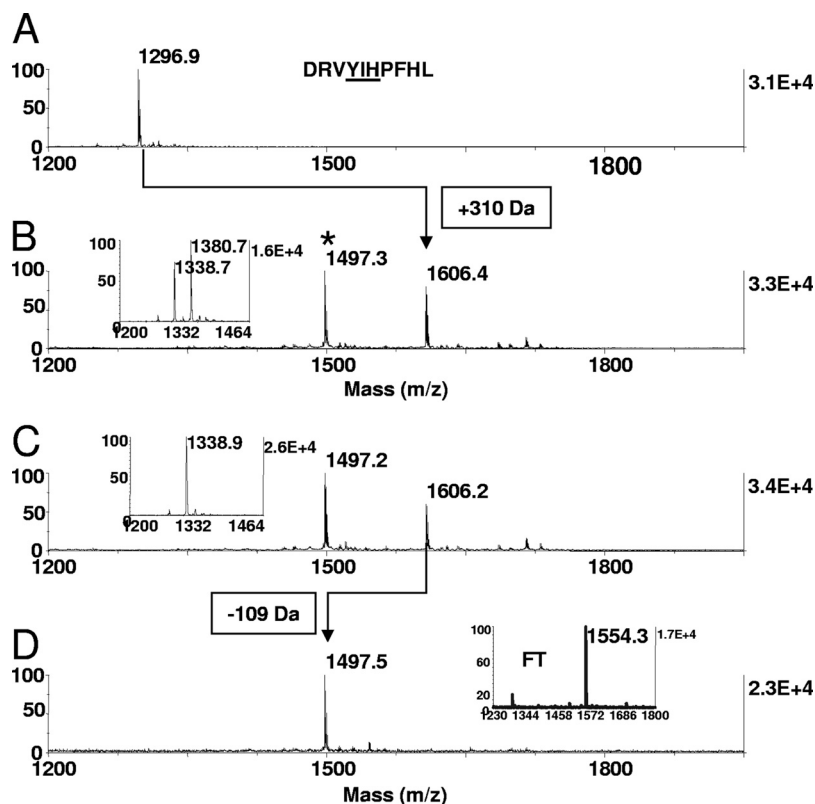Serial in-solution reaction schemes, as practiced in proteomics studies, rely on classical desalting methods,

FIG. 2

Sample preparation at the peptide level: peptide thiolation. Angiotensin I [DRVYIHPFHL, m/z 1296.9 (20 pmoles)], was immobilized on ZipTip$_{C18}$ pipette tips and incubated with 20 mM Sulfo-LC-SPDP in sodium phosphate (pH 8.0) for 30 min at room temperature. The derivative was then exposed for 15 min at 37°C to alkaline 2% hydroxylamine (pH 9.4), followed by incubation in 20 mM sodium phosphate, supplemented with 5 mM TCEP (pH 8.0). MALDI-MS spectra of (A) unmodified peptide; (B) after acylation; (C) after O-deacylation; (D) after 2-pyridyl disulfide group reduction. Cross-arrows indicate the native peptide at m/z 1269.9, its acylation product at m/z 1606.4, and its thiol-functionalized derivative at m/z 1497.5. The mass shifts accompanying the reactions are highlighted with boxes. (B) *, Prereleased thiol derivative. In parallel, the peptide was carried through the three-step, solid-phase reaction scheme using Sulfo-NHS acetate as an acylation reagent. (B and C, insets) Spectrum after acylation and O-deacylation, respectively. Note complete reversal of transesterification at tyrosine upon hydroxylamine treatment. The reactivity-enhancing amino acid triad is underlined in the peptide sequence shown in A. (D, inset) Spectrum of the thiolated peptide recovered from activated thiol sepharose after reaction with iodoacetamide (see text for details). One-tenth of the eluates corresponding to ~2 pmoles peptide was applied to the target.

such as microdialysis, reversed-phase HPLC, and centrifugal sample concentration.[15,16] These handling steps are considered as problematic because of the high potential of adsorptive sample loss. Especially dry-down of the desalted peptides preceding derivatization can cause adsorptive peptide loss, ranging up to 50% of the starting solution after a single evaporation step, as seen with low levels of analyte.[17] Also, the use of reversed-phase pipette tip extraction, used in a more recent proteomics study, was met with limited success.[18] We found that owing to the incompleteness of sample adsorption, an average of 20% of the sample was lost after each purification step.[14] As schematically depicted in Fig. 1C, this cumulative sample loss was avoided by on-phase reagent exchange, eliminating sample transfer between the reaction steps. As a result, the analyte, once

bound to the support, could be carried through the multistep reaction scheme with minimal sample loss, providing a significant advantage in sample-limited situations (Fig. 2) It is noteworthy that the benefits of this reaction format had been recognized more than one decade ago.[19] It has evolved as the predominant sample-preparation technique for trace analysis of bioorganic compounds and found widespread application in pharmaceutical and toxicological studies.[20]

Test peptide and digests solutions used in our study were supplemented with trace amounts of OGS, a MALDI-MS-compatible, nonionic detergent, to minimize peptide adsorption to plastic surfaces during sample handling.[21] The additive has been shown to promote peptide ionization in MALDI-MS, facilitating detection of rela-

tively large species in the mass range between 4 and 11 kDa.[22] To take advantage of this effect, peptide elution from the ZipTip$_{C18}$ pipette tips as well as affinity chromatography on activated thiol sepharose were performed in the presence of the detergent.

Sample Preparation at the Protein Level

As illustrated in Fig. 1A and B, derivatization of the protein involved disulfide reduction and carboxyamidomethylation of the liberated thiols, followed by acetylation of the protein's primary amino groups. As demonstrated with the test protein, both the thiol- and amine-blocking step proceeded to completion. These results are discussed later in the text.

The derivatized samples were then subjected to BEX into 50 mM ammonium bicarbonate/1 M urea (pH 8.0) using the Zeba spin column with a 7 kDa MWCO. The recovered fraction was supplemented with trypsin to a substrate:enzyme ratio of 20:1 and incubated overnight at 37°C. With exception of endoproteinase Lys-C and Lys-N, other urea-compatible endoproteinases (e.g., chymotrypsin, pepsin) may be used to obtain complementary sequence information. The fragmentation reaction was halted by acidification, followed by adsorption of the digest on ZipTip$_{C18}$ pipette tips used as venue for subsequent derivatization.

Sample Preparation at the Peptide Level

Our sample-preparation strategy uses an acylation step to incorporate a disulfide moiety into the N-termini of the (neo) internal and C-terminal fragments. Sulfo-LC-SPDP, a commonly used protein cross-linking agent, was evaluated for this purpose.[23] In its application as a protein thiolation agent, the reagent's NHS-ester moiety is reacted with amines to form an amide linkage. The terminal 2-pyridyldithiol group is then reductively cleaved to form a free sulfhydryl group. To test this sequence of reactions on the solid phase, angiotensin I (DRVYIHPFHL, *m/z* 1296.5), selected as a model peptide, was immobilized on ZipTip$_{C18}$ pipette tips and reacted for 30 min at room temperature, with 20 mM Sulfo-LC-SPDP in a buffered sodium phosphate (pH 8.0), subsequently for 15 min at 37°C with alkaline 2% hydroxylamine (pH 9.4) and finally, for 15 min at 37°C with a 20 mM sodium phosphate solution, supplemented with 5 mM TCEP (pH 8.0). MALDI-MS analysis of the samples showed that the NHS-ester reaction, giving rise to the ion at *m/z* 1606.4 (Fig. 2B) and the formation of the thiol-functionalized counterpart at *m/z* 1497.5, proceeded to near-completion and that these reactions resulted in the expected 201 Da net mass addition (Fig. 2D). Furthermore, the data revealed that during the NHS-ester reaction, a significant portion of the

conjugate was preconverted to its thiol-functionalized analog (Fig. 2B, asterisk). This effect was attributed to reagent synthesis side-products and as such, has no impact on the use of Sulfo-LC-SPDP as a thiolation reagent.

Furthermore, the peptide proved impervious to acylation of the tyrosine hydroxyl side chain (*O*-acylation). In contrast, Sulfo-NHS acetate proved highly effective to *O*-acylate, the peptide's hydroxyl group attributable to the reagent's strong response to the reactivity-enhancement effect of histidine within the YIH amino acid triad, in which isoleucine can be substituted for any amino acid residue and tyrosine for serine or threonine (Fig. 2B, inset).[24] Subsequent treatment with hydroxylamine resulted in regeneration of tyrosine by hydrolysis of the *O*-linked ester bond, eliminating the signal dilution (Fig 2C, inset). This structure-dependent reactivity modulation effect explains the finding that RSA proved prone to *O*-acylation, therefore necessitating the inclusion of *O*-deacylation in the sample-preparation method, as discussed later in this report. Taken together, the results highlight the use of the solid-phase platform to ensure for throughput and optimal efficiency of derivatization.

As noted previously, the C-terminal and internal peptides can be reductively released from the affinity support and used for further manipulation. In this process, the peptides are recovered in their thiolated form and are to be blocked by alkylation to prevent unwanted intermolecular cross-reaction (see Fig. 1). To test this, eluates of the thiol-functionalized test peptide at *m/z* 1497.5 were bound to activated thiol sepharose, reductively released from the resin, and carboxyamidomethylated in situ before collection. MALDI-MS of the material enriched from the flow-through fraction showed the dominant ion at *m/z* 1554.3, corresponding to the peptide's alkylated counterpart (Fig. 2D, inset).

Covalent Chromatography

The method is based on capture of proteins/peptides from a mixture on the activated thiol sepharose by the thiol-disulfide interchange and removal of the nonbound material, followed by reductive release of the disulfide-linked material.[25] In the application of this technique to the isolation of N-terminal fragments, efficient removal of the thiol-functionalized peptides was perceived as a prerequisite to ensure the selective recovery of the desired species. To test the depletion efficiency of the retrieval system, 10 pmoles immobilized [Glu[1]] fibrinopeptide B (EGVND-NEEFFAR, *m/z* 1570.5) was converted to its acetylated counterpart and spiked into 50 pmoles of a tryptic digest prepared from HSA, carbonic anhydrase, β-lactoglobulin, and myoglobin. The mixture was carried through the thiolation reaction scheme, as described above, and submitted
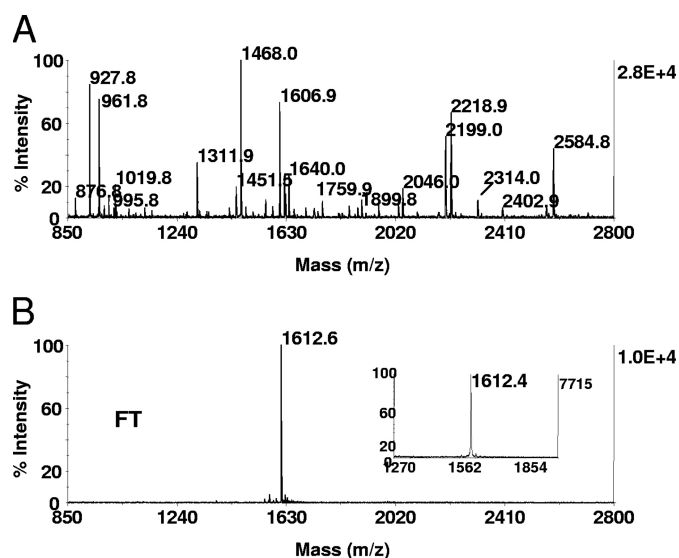
A

B

FIG. 3

Evaluation of microscale covalent chromatography. [Glu[1]] Fibrino-peptide B (EGVNDNEEFFAR, *m/z* 1570.5, 10 pmoles) was adsorbed on ZipTip$_{C18}$ pipette tips exposed to a PBS containing 20 mM Sulfo-NHS acetate for 20 min at 55°C (pH 8.0). A tryptic digest (50 pmoles) prepared from a mixture of HSA, carbonic anhydrase, β-lac-toglobulin, and myoglobin was spiked with the acetylated peptide (serving as an N-terminally blocked peptide mimic), carried through the thiolation reaction cycle described in Fig. 2, and subjected to covalent chromatography. The starting material and the flow-through fraction were analyzed by MALDI-MS. (A) Unmodified material and (B) flow-through fraction MALDI-MS spectra. Note the efficiency of peptide depletion. One-twentieth of the eluates corresponding to ∼2.5 pmoles peptide was applied to the target. (B, inset) Spectrum of displaced peptide at 0.5 pmole sample load. The peptide was concentrated from the flow-through on a ZipTip$_{μ-C18}$ pipette tip and deposited in the matrix onto the target.

A

B

FIG. 4

N-Terminal peptide isolation from whole protein. Tryptic digests generated from carboxyamidomethylated-acetylated, intact RSA (25 pmoles) were adsorbed on ZipTip$_{C18}$ pipette tips and carried through the thiolation reaction cycle described in Fig. 2. The thiol group-functionalized digests were submitted to covalent chromatography. MALDI-MS spectra of (A) carboxyamidomethylated digest; (B) material enriched from the flow-through fraction. (A and B) Asterisks indicate carboxyamidomethylated peptides. The peptide isolate at *m/z* 1261.6 was aligned by database search to the N-terminal tryptic fragment EAHKSEIAHR, spanning residues 25–34. (A) The arrow designates the ion at *m/z* 1177.6, recognized as the isolate's structural counterpart containing uncleaved lysine. Note selectivity of peptide retrieval. One-tenth of the eluates corresponding to ∼2.5 pmoles digest was applied to the target.

to covalent chromatography. MALDI-MS analysis of the samples illustrated in Fig. 3A and B showed that the flow-through fraction was devoid of the digestion products, indicating that they were fully depleted. The affinity resin was then subjected to an additional cycle of the organic solvent wash. Negligible amounts of the acetylated peptide were found in the flow-through fraction, indicating that the N-terminal peptide mimic was effectively excluded from the affinity support (results not shown). Taken together, the data validate the use of the miniaturized covalent chromatographic system to recover selectively amine-blocked peptides with minimal sample loss.

Comparable results were obtained when the protein digest was spiked with 0.5 pmole of the N-terminally blocked peptide, processed as described above, and submitted to covalent chromatography. The peptide was enriched from the flow-through fraction on a ZipTip$_{μ-C18}$ pipette tip and deposited in the matrix onto the target. The high quality of the spectrum underscores the potential to extend
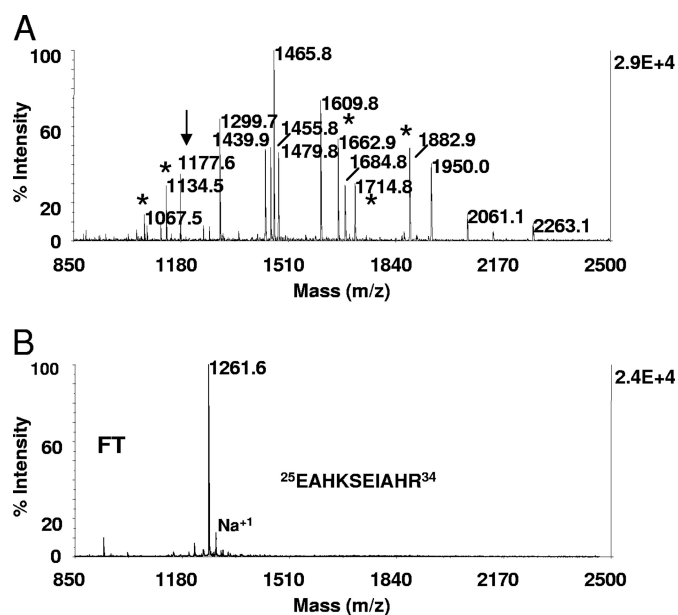
the detection limits of the procedure (Fig. 3B, inset). On the basis of these results, we anticipate that 1 pmole digests should be amenable to N-terminal peptide selection (see below).

Application of Sample-Preparation/Affinity Isolation to Whole Protein

To test the applicability of the N-terminal isolation strategy with whole protein, RSA was reduced, alkylated, and acetylated in the one-pot reaction mode and subsequently digested with trypsin. The digest was then carried through the sequential solid-phase reactions, as described in Fig. 2, and submitted to covalent chromatography. MALDI-MS analysis of the flow-through fraction revealed the prominent ion at *m/z* 1261.6, and there was essentially no background contamination, indicating that the internal and C-fragments were scavenged effectively by the affinity support (Fig. 4B). The isolate aligned within the known protein sequence to the N-terminal fragment, spanning resi-

dues 25–34 and corresponding to EAHKSEIAHR. The structural analog of this peptide could be readily traced at *m/z* 1177.6 as the tryptic miscleavage product in the mass map prepared from the carboxyamidomethylated protein (Fig. 4A, arrow). The spectrum acquired from the acetylated protein digest was devoid of ions corresponding to unreacted species, providing evidence that the amine protection at the protein level reached completion (data not illustrated). We note here that the EAHKSE sequence tag unambiguously identified the protein, along with rabbit and mouse serum albumin, among the 585,169 protein entries contained in the UniProtKB/Swiss-Prot database (release 2014_2 of Feb 14), underscoring the extraordinary high sequence specificity at the protein N-termini. Taken together, the data validate the use of the miniaturized sample processing/covalent chromatographic system to isolate selectively N-terminal proteolytic fragments from whole protein.

Furthermore, we anticipate that digest prepared from protein mixtures, such as that derived from isolated protein complexes, should be readily processed by this procedure. Scale-up of the method will be required to fully exploit the method's inherent sample simplification capability for proteome-wide applications. To this purpose, we have begun to explore the use of reversed-phase spin columns, which accommodate up to 300 μg digests.

Application of Sample-Preparation/Affinity Isolation to Gel-Separated Protein

With the use of gel-separated RSA, we next developed an in-gel protocol of the protein sample-preparation method. We reasoned that this format would be advantageous, as proteins are presented frequently in gel-separated form for subsequent in-gel proteolytic digestion, and has been shown to accommodate relatively low-level amounts of protein (<2 pmoles).

In the experiments, GelCode Blue-stained bands from 25 pmoles RSA loaded onto the gels were destained, carboxyamidomethylated, and subsequently reacted with 20 mM Sulfo-NHS acetate in 50 mM sodium phosphate buffer (pH 8.0) with agitation. After 2 h incubation at 37°C, bands were briefly washed and subjected to in-gel tryptic digestion, allowed to proceed overnight at 37°C at a substrate:enzyme ratio of 10:1. Gel extracts were immobilized on ZipTip pipette tips and carried through the sequence of solid-phase reactions, as described in Fig. 2. Eluates were processed by covalent chromatography as outlined above. Tryptic digest prepared from the acetylated protein along with the material retrieved from the affinity support were analyzed by MALDI-MS. The spectrum acquired from the flow-through fraction displayed the prominent ion at *m/z* 1261.6. The recovered peptide was recog-
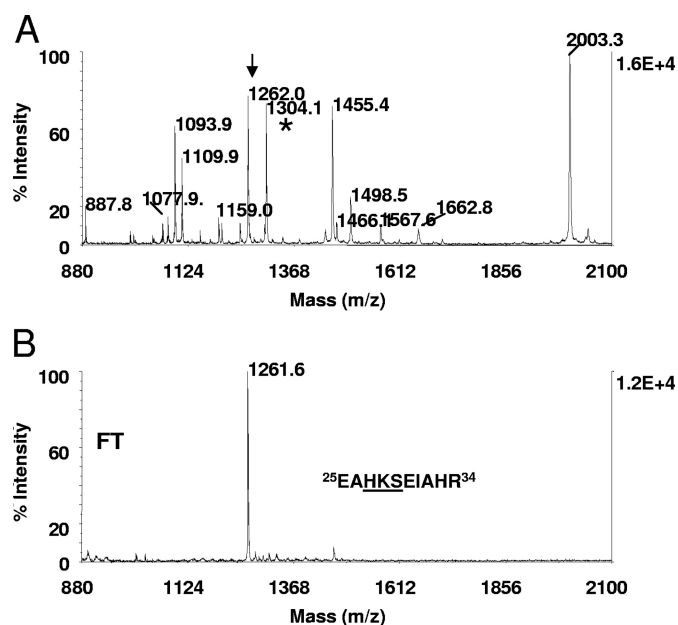

FIG. 5

N-Terminal peptide isolation from gel-separated protein. RSA (25 pmoles loaded onto the gel) was subjected to in-gel carboxyamidomethylation, acetylation followed by tryptic digestion. Gel extracts were bound to ZipTip$_{C18}$ pipette tips, thiolated as described in Fig. 2, and submitted to covalent chromatography. MALDI-MS spectra of (A) digest after acetylation; (B) material enriched from the flow-through fraction. (A) Arrow and asterisk designate the N-terminal species and its *O*-acylated counterpart at *m/z* 1304.1, respectively. (B) The prominent ion matched by database search to the N-terminal isolate EAHKSEIAHR, spanning residues 25–34. The reactivity-enhancing amino acid triad is underlined in the peptide sequence. Note efficiency of *O*-acylation reversal by hydroxylamine, preventing signal dilution, and selectivity of peptide retrieval. One-tenth of the eluates corresponding to ~2 pmoles digest was applied to the target.

nized by a database search as the N-terminal isolate, corresponding to [25]EAHKSEIAHR[34] (Fig. 5B). Coisolates, which would arise from incomplete peptide depletion on activated thiol sepharose, were not observed in the spectrum. Furthermore, the N-terminal peptide could be readily traced in the spectrum prepared from the tryptic digest of the acetylated protein and was accompanied by the ion at *m/z* 1304.1 (Fig. 5A, asterisk). The difference mass of 42 Da between this ion pair, ionizing at comparably high intensity, indicates that *O*-transesterification had occurred at the protein level promoted by histidine within the HKS amino acid triad.[24] The data revealed that the *O*-deacylation step at the peptide level ensured that the isolate was recovered, undiluted from the affinity support. Taken together, the results validate the use of the gel-based sample-preparation protocol/covalent chromatographic system to isolate selectively N-terminal proteolytic fragments from gel-separated protein.

CONCLUSIONS

A sample-preparation method for N-terminal peptide isolation from protein digests has been developed. The method uses a series of chemical reactions at the protein and peptide level, which was optimized for throughput and completeness of derivatization. The use of reversed-phase supports as a venue for peptide derivatization proved instrumental to recover the reaction products with minimal sample loss. The N-terminal peptide was effectively selected by covalent chromatography. The microscale sample-preparation method/covalent affinity retrieval system combines robustness with simplicity of operation using standard equipment readily available in most biological laboratories and is expected to be readily adaptable to proteome-wide applications.

DISCLOSURE

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